

METHOD FOR DETERMINING INTERACTION OF A POLYPEPTIDE WITH A POTENTIAL BINDING PARTNER

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 [0001] This application claims the benefit of priority of Singapore patent application No. 201303835-1 filed on 16 May 2013, the content of which is incorporated herein by reference in its entirety for all purposes.

TECHNICAL FIELD

- 10 [0002] The present invention refers to the field of biochemistry and in particular to technologies concerning determination of interaction between a polypeptide and a potential binding partner.

BACKGROUND

- 15 [0003] In drug discovery, high throughput screening of low molecular weight ligand to protein is of importance to simplify and to expedite drug discovery processes. In structural biology, study of protein stability and ligand induced protein stabilization are important in understanding thermal stability of protein, improving protein purification, identifying lead compounds in drug discovery programs, as well as facilitating the protein structural
20 elucidation, especially in crystallization.

- [0004] Current methods available for detecting ligand binding and protein stability include X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR), differential scanning fluorimetry (DSF), isothermal titration calorimetry (ITC), isothermal denaturation (ITD), surface plasmon resonance spectroscopy (SPR), circular dichroism (CD), fluorescence
25 polarization (PF), and protein aggregation-based differential static light scattering (DSLS).

- [0005] For example, although X-ray crystallography and NMR remain the most powerful tools in providing structural information, not all targets give diffractable crystals with useful resolution, and the latter approach is typically restricted to small targets that are less than 50 kDa. Also, despite existing use of DSF and ITD to study protein thermal stability, they are
30 unsuitable for proteins that (i) have high fluorescence background, (ii) are partially purified, or (iii) do not aggregate upon unfolding.

[0006] Green fluorescent protein based stability assay (GFP-Basta) is a relatively new method which studies thermal stability of fused GFP-protein of interest using GFP as probes. The assay, however, depends on GFP stability and is therefore not applicable for proteins that are more stable than GFP. Further, despite it being a considerably stable protein, GFP suffers from fluorescence signal loss and restricted storage conditions.

[0007] In view of the above, there remains a need for an improved method for determining interactions between polypeptides such as proteins, and potential binding partners of the polypeptides that overcomes or at least alleviates one or more of the above-mentioned disadvantages.

SUMMARY OF THE INVENTION

[0008] In a first aspect, the invention refers to a method for determining interaction of a polypeptide with a potential binding partner. The method comprises

- a) providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles;
- b) subjecting the mixture to at least one of (i) an electrolyte and (ii) temperature increase; and
- c) determining the interaction of the polypeptide with the potential binding partner by determining degree of aggregation of the metallic nanoparticles.

[0009] In a second aspect, the invention refers to a method for identifying a binding partner for a polypeptide of interest. The method comprises

- a) contacting the polypeptide and a candidate binding partner with metallic nanoparticles under conditions that allow polypeptide-nanoparticle conjugate formation;
- b) subjecting the conjugate to at least one of (i) an electrolyte and (ii) temperature increase; and
- c) determining degree of aggregation of the metallic nanoparticles, wherein a decreased aggregation of the metallic nanoparticles compared to a reference with no binding partner indicates binding of the polypeptide and the candidate binding partner.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

5 [0011] **FIG. 1A** is a schematic diagram of the assay principle according to embodiments. Polypeptides such as protein having one or more binding partners or ligands bound thereto (I) have higher salt and thermal stability, thus providing better protection to metallic nanoparticles in the presence of salt and/or under heat treatment. In comparison, polypeptides which do not have binding partners bound thereto, tend to denature under high salt content
10 (II), thereby providing moderate protection to the metallic nanoparticles. Likewise, polypeptides which do not have binding partners bound thereto are largely denatured under heat treatment (III), providing low protection to metallic nanoparticles. **FIG. 1B** shows exemplary UV-vis spectra generated according to an embodiment, which may be used as a measure of metallic nanoparticles aggregation profile.

15 [0012] **FIG. 2** shows schematic diagrams of (A) room temperature and (B) thermal treatment assay procedures according to various embodiments.

[0013] **FIG. 3A** is a graph showing UV-vis spectra of (a) citrates-coated AuNPs, (b) FoxA1-coated AuNPs, (c) AuNPs with salt and (d) FoxA1-coated AuNPs with salt. **FIG. 3B** is a graph showing UV-vis spectra of AuNPs with FoxA1 loaded with various ligands. Lig 1-
20 2 are positive ligands whereas lig 3-5 are negative ligands. **FIG. 3C** is a graph summary of dispersion status measured as A_{540}/A_{600} for FoxA1-AuNPs with positive ligands (lig1 and lig2) and negative ligands (lig3 to lig9). Two controls are also shown. The final concentration of sodium chloride (NaCl) in these experiments is 86 mM.

[0014] **FIG. 4A** is a graph showing UV-vis spectra of AuNPs with FoxA1-ligand solution
25 treated at 60 °C. **FIG. 4B** is a graph showing dispersion degree of the respective AuNPs solution, measured as A_{540}/A_{700} . Both color and spectra show greater differentiation between positive and negative ligands, as compared to room temperature (RT) results as shown in **FIG. 3B**.

[0015] **FIG. 5** is a graph showing UV-vis spectra of AuNPs with AP-2 γ , (A) without; and
30 (B) with heat treatment at 60 °C. UV-vis spectra of AuNPs with (C) positive (lig 2) and (D) negative ligand (lig 3) loaded-AP2 γ under 60 °C treatment are also shown.

[0016] **FIG. 6A** is a graph showing UV-vis spectra of FoxA1-coated AuNPs titrated with increased concentration of lig 2 (Dawson). **FIG. 6B** shows Michaelis-Menten plot of A_{540}/A_{700} vs ligand concentration.

[0017] **FIG. 7** is a graph showing Michaelis-Menten plot of Surface Plasmon Resonance (SPR) angle shift vs. ligand concentration.

[0018] **FIG. 8** is a graph showing plot of temperature scan denaturation curve for FoxA1 and its ligand complex. As the temperature increases, A_{700}/A_{540} ratio has increased significantly, indicating the protein-unfolding on AuNPs surface that caused absence of electrosteric protection to the AuNPs.

[0019] **FIG. 9A** is a schematic diagram of the interaction of ligands with AuNPs. Some ligands may cause AuNPs aggregation. **FIG. 9B** shows UV-vis spectra of AuNPs mixed with ligands 1-9. **FIG. 9C** shows respective color photographs of the samples.

DETAILED DESCRIPTION OF THE INVENTION

[0020] The method disclosed herein is based on the finding that interaction between a polypeptide and potential binding partner, which may be in the form of polypeptide-binding partner complex, may stabilize negatively charged metallic nanoparticles against electrolyte-induced and/or thermal-induced aggregation to a larger extent compared to the polypeptide alone, or in presence of a polypeptide and a potential binding partner that does not interact with the polypeptide. It has been shown herein that degree of aggregation of the metallic nanoparticles is dependent on binding affinity between the polypeptide and the potential binding partner. Differences in binding affinity between potential binding partners with polypeptides, as well as absence of potential binding partners in a sample translate into differences in extent of stabilization of negatively charged metallic nanoparticles against electrolyte-induced and/or thermal-induced aggregation. In addition, methods disclosed herein allow determination of parameters of polypeptide-binding partner binding events, such as binding constant (K_d) and temperature of aggregation (T_{agg}) measurement, in a cost effective, simple and efficient manner.

[0021] In a first aspect, a method for determining interaction of a polypeptide with a potential binding partner is provided. The term “determine” refers to any qualitative and quantitative identification, such as identification of presence or absence of interaction between a polypeptide with a potential binding partner, strength of the interaction, or amount

of the interaction, that may be determined using the method described herein. Quantitative identification as used herein may be carried out via correlation of properties with that of a known sample. For example, quantitative identification of interaction between a polypeptide with a potential binding partner, such as amount of interaction, may be determined with reference to the corresponding amount of interaction of the sample.

[0022] The term “interaction” refers to the properties of binding or attraction between two entities. It refers to any kind of chemical bonding, such as covalent bond, hydrogen bond or ionic bond, and any kind of physical bonding, such as dipole-dipole, hydrophobic interaction, or Van der Waals forces. Accordingly, interaction between a polypeptide with a potential binding partner refers to the properties of binding or attraction between the polypeptide and the potential binding partner.

[0023] The term “polypeptide” as used herein refers generally to a single chain amino acid polymer of more than 100 amino acid monomers. Peptides refer generally to amino acid dimers (dipeptides), oligomers (oligopeptides) of up to about 25 to 50 amino acids, and short polymers of about 2 to 100 amino acids in length. The term “protein” as used herein refers generally to a 3D-structure of one or more polypeptide chains that may be non-covalently or covalently (via disulfide bridges) be associated with each other.

[0024] Amino acids are molecules containing at least one carboxyl group ($-\text{COOH}$) and one amine ($-\text{NH}_2$) group. A side chain is present on the amino acid molecule, which can affect properties such as polarity and acid-base properties of the amino acid. The side chain can vary in size from a hydrogen atom in glycine, to a phenyl group in phenylalanine. Further examples of amino acids include aspartic acid, glutamic acid, arginine, lysine, asparagine, glutamine, alanine, tryptophan as well as any non-standard amino acid, such as selenocysteine, lanthionine, 2-aminoisobutyric acid, dehydroalanine or gamma-aminobutyric acid; or amino acid derivatives, such as 5-hydroxytryptophan or L-dihydroxyphenylalanine. Therefore, a protein molecule is made from a long chain of these amino acids, each linked to its neighbor through a covalent peptide ($-\text{CONH}-$) bond.

[0025] Proteins may be classified into five major categories, i.e. structural proteins, catalytic proteins, transport proteins, protective proteins, and regulatory proteins.

[0026] Structural proteins refer to proteins that maintain structures of other biological components, like cells and tissues. Examples of structural proteins include, but are not limited to, collagen, keratin, elastin, sklerotin, and fibroin. Catalytic proteins refer to proteins that

catalyze chemical and biochemical reactions within and outside of living cell. An example of catalytic protein is enzyme. Transport proteins refer to proteins that functions to convey molecules into and out of a cell, as well as transportation of molecules intracellularly. Examples of transport proteins include, but are not limited to, hemoglobin and serum albumin. Protective proteins refer to proteins that have the ability to protect other proteins from damage. In general, protective proteins function by minimizing degradation via conformational changes and enzymatic cleavage or digestion, thereby enhancing cell viability in the cells, tissues and organizations from which they are derived. Examples of protective proteins include, but are not limited to antibodies and thrombin. Regulatory proteins refer to proteins that bind to specific regulatory sequences of nucleic acids and act to switch genes on and off and thereby regulate the transcription of genes. Examples of regulatory proteins include, but are not limited to, hormones such as estrogen, adrenalin, insulin, growth hormone, steroid hormone, and thyroid hormone.

[0027] Proteins may be produced by living cells, and the activities of all living cells are conducted primarily by the thousands of different types of proteins each cell produces. The blueprint or code for synthesizing each protein may be found in a corresponding gene, i.e. each gene encodes the information needed to synthesize a specific protein. Gene expression may result in production of the protein, by transcription of the gene by RNA polymerase to produce a messenger RNA (mRNA) that contains the same protein-encoding information, and translation of the mRNA by ribosomes to produce the protein.

[0028] In various embodiments, the polypeptide is selected from the group consisting of an antibody, a protein receptor, and a transcription factor. In specific embodiments, the polypeptide comprises or consists of a transcription factor. The term “transcription” refers to the process of copying a DNA sequence of the gene by RNA polymerase into the mRNA, using the DNA as a template, while the term “transcription factor” as used herein refers to a polypeptide that binds to specific DNA sequences, thereby controlling the flow (or transcription) of genetic information from DNA to messenger RNA.

[0029] The transcription factor may be selected from the group consisting of ERalpha, ERbeta, FoxA1, and AP-2γ. In specific embodiments, the transcription factor comprises or consists of FoxA1 or AP-2γ.

[0030] FOXA1 (Forkhead box protein A1), which may otherwise be termed as hepatocyte nuclear factor 3-alpha (HNF-3A), refers to a protein having an amino acid sequence

substantially identical to the FoxA1 sequence of GenBank Accession No. NP—004487. AP-2 γ (activating enhancer binding protein 2 gamma), which may otherwise be termed as transcription factor AP-2 gamma (TFAP2C), refers to a protein having an amino acid sequence substantially identical to the AP-2 γ sequence of GenBank Accession No. BAC11805. Both FOXA1 and AP-2 γ are important oncogenic factors pivotal in regulating the transcriptional activity of estrogen receptor alpha and controlling the expression of estrogen-responsive breast cancer cells, for example.

[0031] The method disclosed herein comprises providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles. The method does not require that any of the molecules involved in the method is attached to a solid phase. For example, all components used may be dispersed in a liquid or aqueous solution. Further, the method may advantageously be carried out at ambient conditions, such as at room temperature.

[0032] As used herein, the term “binding partner”, otherwise termed herein as a “ligand”, refers to a moiety, which may be in the form of an atom, an ion or a molecule, that is able to bind to a specific binding site on a polypeptide. The term “potential binding partner” or “candidate binding partner”, on the other hand, refers to a moiety, which may be in the form of an atom, an ion or a molecule that may or may not bind to a specific binding site on a polypeptide. An example of potential binding partners includes small molecule drugs.

[0033] The binding partner that interacts with the polypeptide may depend on the polypeptide that is used. In various embodiments, the potential binding partner is a low molecular weight ligand or another protein. For example, the ligand may be a low molecular weight ligand having a molecular weight in the range of about 100 to about 3500, such as about 100 to about 3000, about 100 to about 2500, about 100 to about 2000, about 100 to about 1500, about 100 to about 1000, about 100 to about 600, about 1000 to about 3500, about 1500 to about 3500, about 2000 to about 3500, about 2500 to about 3500, about 2000 to about 3000, or about 1500 to about 2500.

[0034] In various embodiments, potential binding partners are selected from the group consisting of Quinobene, Dawson, [[[Z)-(3-oxopyridin-2-ylidene)methyl]amino]thiourea, 8-amino-10-phenylphenazin-2-one, 2-bromo-1H-phenalen-1-one, Lomofungin, N,N-dimethyl-daunorubicin, 6H-Imidazo[4,1-de]acridin-6-one, Quinacrine, and derivatives thereof.

[0035] As mentioned above, the binding partner that interacts with the polypeptide may depend on the polypeptide that is used. When the polypeptide is FoxA1, for example, binding partners that interact with the polypeptide may include Quinobene and Dawson polyoxometalate (POM).

5 [0036] The mixture comprising the polypeptide and the potential binding partner also comprises metallic nanoparticles.

[0037] To enhance the measurability of changes using a method disclosed herein, the metallic nanoparticles may be plasmonic nanoparticles. Plasmonic nanoparticles refer to metallic nanoparticles whose surface plasmon resonance frequency depends on the
10 nanoparticle size, shape and composition. Light incident on plasmonic nanoparticles induces the conduction electrons in them to oscillate collectively with a certain resonant frequency. This resonant frequency changes in case the metallic nanoparticles aggregate and thus may be measured, such as with optical methods.

[0038] Although silver and gold are the most commonly used materials for plasmonic
15 nanoparticles, theoretically any metal, alloy or semiconductor with a large negative real dielectric constant and small imaginary dielectric constant may be used. In some embodiments, the nanoparticles are noble metal particles, alloys of noble metal particles or mixtures of materials referred to herein. Noble metal includes silver (Ag), palladium (Pd), gold (Au), platinum (Pt), iridium (Ir), osmium (Os), rhodium (Rh), and ruthenium (Ru).
20 Examples of noble metal alloys include alloys of platinum and iridium, Pd-Pt, Pd-Rh or Pd-Pt-Rh, to name only a few. Other materials, such as aluminium, potentially offer advantages in refractive index sensitivity, different surface chemistries, and resonances into the ultraviolet, where many organic molecules absorb light. Copper may also be used as possible metal for the metallic nanoparticles.

25 [0039] In specific embodiments, the noble metal nanoparticles comprise or consist of a noble metal selected from the group consisting of silver, gold, and alloys of the aforementioned materials.

[0040] The nanoparticles may be in any known shape. For example, in some
30 embodiments, the nanoparticles have a shape which is selected from the group consisting of a nanosphere, a nanocube, a nanorod, a nanotube, a nanostar, a nanocrescent, a nanoplate, and mixtures thereof. With mixtures thereof, it is meant that the nanoparticles used may include

nanoparticles having not only a tubular shape, but also nanoparticles having a plate like shape. In some embodiments, nanoparticles with different shapes are used.

[0041] In various embodiments, the metallic nanoparticles each have a size in a range of about 10 nm to about 100 nm. As the nanoparticles may not be regular in shape, size of a nanoparticle is defined by a maximal length of a line segment passing through the centre and connecting two points on the periphery of a nanoparticle.

[0042] For example, the metallic nanoparticles may have a size in a range of about 10 nm to about 80 nm, about 10 nm to about 60 nm, about 10 nm to about 50 nm, about 10 nm to about 30 nm, about 30 nm to about 100 nm, about 30 nm to about 70 nm, about 30 nm to about 50 nm, about 50 nm to about 100 nm, about 50 nm to about 80 nm, about 50 nm to about 70 nm, about 70 nm to about 100 nm, about 30 nm to about 80 nm, about 30 nm to about 60 nm, or about 40 nm to about 70 nm.

[0043] The metallic nanoparticles may be well dispersed. In some embodiments, methods such as agitation or stirring may be used to disperse the metallic nanoparticles. In various embodiments, the metallic nanoparticles are at least substantially homogeneously dispersed in the mixture.

[0044] The metallic nanoparticles may, in some embodiments, be negatively charged metallic nanoparticles. The negatively charged metallic nanoparticles may be used to avoid aggregation of the metallic nanoparticles prior to determination of interaction of polypeptide with potential binding partner. For example, the negatively charged metallic nanoparticles may be metallic nanoparticles carrying a negative charge at nanoparticle surface.

[0045] Without wishing to be bound by theory, it is postulated that the negatively charged metallic nanoparticles, which behave like colloidal particles, are stabilized in solution due to electrostatic stabilization. Electrostatic stabilization refers to the mechanism in which the attraction van der Waals forces between the particles are counterbalanced by the repulsive Coulomb forces acting between the negatively charged particles. In this mechanism, the particles may have an electric double layer, which is electrically neutral, and which consists of three parts. The first part called a surface charge layer is the negatively charged ions on the particle surface. The second part called a stern layer is made up of positive ions adsorbed on the particle surface due to electrostatic attraction. The third part comprises a diffuse layer made up of both positive and negative ions present in the solution. Presence of the electric double layer on the particles results in stabilization of the particles such that they do not

aggregate. Depending on the type and extent of interaction between a polypeptide and a potential binding partner, stability of the metallic nanoparticles may be changed by addition of the polypeptide and the potential binding partner.

[0046] Metallic nanoparticles with a negative surface charge may be nanoparticles wherein the negative charge of the metallic nanoparticles is conferred by a carboxylic acid or sulfonic acid or carbolic acid or a mixture of the aforementioned acids which is immobilized at the surface of the metallic nanoparticles.

[0047] In various embodiments, the carboxylic acid may be, but is not limited to citric acid, lactic acid, acetic acid, formic acid, oxalic acid, uric acid, pyrenedodecanoic acid, mercaptosuccinic acid, aspartic acid, to name only a few. Example of a method to immobilize such acids at the surface of metallic nanoparticles is thermal reduction of HAuCl_4 with sodium citrate.

[0048] Providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles may comprise contacting the polypeptide with the potential binding partner, and subsequently contacting the resulting mixture with the metallic nanoparticles.

[0049] In various embodiments, providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles comprises contacting the polypeptide with the metallic nanoparticles, and subsequently contacting the resulting mixture with the potential binding partner.

[0050] In some embodiments, providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles comprises contacting the potential binding partner with the metallic nanoparticles, and subsequently contacting the resulting mixture with the polypeptide.

[0051] In further embodiments, providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles comprises simultaneous contacting of the polypeptide, the potential binding partner, and the metallic nanoparticles.

[0052] Providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles may comprise mixing or dispersing the polypeptide, potential binding partner and metallic nanoparticles in a suitable solvent, such as a buffer solution. All kinds of buffers may be used to conduct the method disclosed herein, such as a tris(hydroxymethyl)aminomethane (TRIS) buffer, or a tris-hydrochloric acid buffer (TRIS-HCl), or a phosphate buffered saline (PBS), to name only a few. In various embodiments, the

pH of the solution in which the method disclosed herein is carried out is neutral (i.e. about pH 7).

[0053] In various embodiments, the polypeptide, potential binding partner and metallic nanoparticles in the mixture are incubated for a period of time so as to allow sufficient time for the polypeptide and potential binding partner to interact. A suitable amount of time may be dependent on reaction conditions, such as the type of polypeptide and potential binding partner, solvent used, temperature of solution, and amount of solution. A person skilled in the art would be able to determine the appropriate amount of time for any interaction that may take place to occur between the polypeptide and potential binding partner. Typically, the amount of time for interaction to take place between the polypeptide and potential binding partner is in the order of minutes, for example about 5 minutes, about 10 minutes, about 20 minutes, or about 30 minutes.

[0054] The potential binding partner may be in any suitable amount and ratio to the polypeptide, or be present in excess in the mixture. In some embodiments, the molar ratio of polypeptide and potential binding partner in the mixture may be about 1:4.

[0055] The method further comprises subjecting the mixture to at least one of (i) an electrolyte and (ii) temperature increase.

[0056] The term "electrolyte" as used herein refers to ionic or molecular substances which, when in solution, break down or disassociate to form differently charged ions or differently charged particles. Addition of the electrolyte may compress the double layer around a metallic nanoparticle. When the double layer is compressed, the sphere of influence of the charges on the particle is reduced, allowing it to approach closer to another particle. This can allow the particles to combine, leading to aggregation of the metallic particles, which can show up as a change in color of the solution. Generally, stability of the solution is in the order of the following color sequence, i.e. red > purple > blue > grey, with red being the most stable and grey being the least stable. Examples of an electrolyte include, but are not limited to, an organic acid, an inorganic acid, a base, and a salt.

[0057] Non-limiting examples of an organic acid include carboxylic acids as described herein, sulphonic acids such as butanesulphonic acid, butanedisulphonic acid, benzenesulphonic acid, methylbenzenesulphonic acid, ethylbenzenesulphonic acid, dodecylbenzenesulphonic acid, 2,4,6-trimethylbenzenesulphonic acid, 2,4dimethylbenzenesulphonic acid, 5-sulphosalicylic acid, 1-sulphonaphthalene, 2-

sulphonaphthalene, hexanesulphonic acid, octanesulphonic acid and dodecanesulphonic acid,
 and amino acids such as glycine, alanine, valine, α -aminobutyric acid, γ -aminobutyric acid,
 alanine, taurine, serine, ϵ -amino-nhexanoic acid, leucine, norleucine and phenylalanine. Non-
 limiting examples of an inorganic acid include hydrochloric acid, sulfuric acid, sulfurous
 5 acid, nitric acid, nitrous acid, phosphoric acid, boric acid and carbonic acid. Non-limiting
 examples of a base include potassium carbonate, calcium carbonate, sodium carbonate,
 barium carbonate, zinc carbonate hydroxide hydrate, magnesium carbonate hydroxide
 hydrate, calcium hydroxide, sodium hydroxide, magnesium hydroxide and aluminum
 hydroxide.

[0058] In some embodiments, the electrolyte is a salt. Suitable salts that may be used
 herein include any organic and inorganic salt. Organic salts are also known as
 polyelectrolytes or polysalts. Organic salt refers to a complex formed by charge attraction
 between oppositely charged polymers. Accordingly, organic salts are polymers comprising
 cationic and anionic components. An organic salt may be formed by any method well known
 15 in the art, such as by blending aqueous solutions containing the cationic polymer component
 and anionic polymer component, and allowing the charge interaction to occur. For example,
 the cationic polymer component may be a polyamine. Examples of polyamine includes C_1 -
 C_{12} dialkyl diallyl ammonium polymers, such as olydimethyl diallyl ammonium chloride,
 polydiethyl diallyl ammonium chloride, polydimethyl diallyl ammonium bromide,
 20 polydiethyl diallyl ammonium bromide, and poly(dimethyl diallyl ammonium chloride-co-
 acrylamide). For example, the anionic polymer component may be a carboxylic anionic
 polymer, a polysulfonic acid or an alginic acid.

[0059] An inorganic salt may be formed from the neutralization reaction of an acid and a
 base. Examples of inorganic salts include, but are not limited to NaCl, KCl, $CaCl_2$, $BaCl_2$,
 25 $MgCl_2$, NaBr, KBr, NaI, KBr, $NaNO_3$, KNO_3 , $Mg(NO_3)_2$, $Ca(NO_3)_2$, Na_2SO_4 , K_2SO_4 ,
 $NaClO_4$, NH_4Cl , NH_4NO_3 , $(NH_4)_2SO_4$, CH_3COONa , CH_3COONH_4 , to name only a few. At
 least one type of salt may be added. When two or more salts are added, they may be different
 types. For example, Na_2SO_4 may be added with NaCl.

[0060] In various embodiments, the electrolyte comprises or consists of sodium chloride.
 30 Sodium chloride (NaCl) may dissociate in water to form a cation of Na^+ and an anion of Cl^- .
 The concentration of the salt may be in the range of between about 0.01 M to about 0.5 M, or
 between about 0.01 M to about 0.25 M, or between about 0.01 M to about 0.1 M. The amount

of salt required to induce particle aggregation may be determined using UV-Vis spectroscopy, which may be used to characterize particle aggregation extent through spectrum shift.

[0061] In various embodiments, the electrolyte is added to a mixture comprising a polypeptide and a potential binding partner, followed by addition of the metallic nanoparticles to determine interaction between the polypeptide and the potential binding partner. For example, the electrolyte may be added to either the polypeptide or the potential binding partner, prior to contacting the polypeptide with the potential binding partner. Metallic nanoparticles may subsequently be added upon addition of the electrolyte and upon contacting of the polypeptide with the potential binding partner.

[0062] The method may generally be carried out at ambient temperature, which generally refers to a temperature of between about 20 °C to about 40 °C. Even though the method may generally be carried out at ambient conditions, the method according to various embodiments may further comprise heating the mixture so as to subject the mixture to a temperature increase.

[0063] Heating of the mixture may be carried out prior to addition of metallic nanoparticles, so as to improve sensitivity of the method. For example, thermal treatment of polypeptides may cause the polypeptides to misfold to a larger extent and weaker its protection effect on metallic nanoparticles. Consequently, this allows a more intense difference in color presentation between solutions containing binding partners, and solution containing potential binding partners that do not interact with polypeptides, upon addition of metallic nanoparticles. In various embodiments, subjecting the mixture to temperature increase comprises heating the mixture at a temperature in the range of about 25 °C to about 90 °C, such as about 25 °C to about 60 °C, about 25 °C to about 40 °C, about 40 °C to about 90 °C, about 60 °C to about 90 °C, about 30 °C to about 50 °C, or about 35 °C to about 60 °C.

[0064] Heating of the mixture may also be carried out after addition of metallic nanoparticles. In these embodiments, determining degree of aggregation of the metallic nanoparticles, such as generating UV-vis spectrum of the metallic nanoparticles, may be carried out prior to and following the heating to determine presence of potential binding partners.

[0065] The method includes determining the interaction of the polypeptide with the potential binding partner by determining degree of aggregation of the metallic nanoparticles.

Degree of aggregation of the metallic nanoparticles referred to herein may be dependent upon binding affinity between the polypeptide and the potential binding partner. As used herein, “binding affinity” refers to strength of interaction between two entities. Binding affinity is sometimes referred to as K_d or binding constant, which is an indication of the strength of binding between two entities in terms of how easy it is to separate them.

[0066] In various embodiments, the binding constant may be determined by varying concentration of potential binding partners in the mixture, and measuring resultant aggregation degree of metallic nanoparticles. The aggregation degree of metallic nanoparticles may be represented by a ratio in absorbance of metallic nanoparticles in their dispersed state as compared to absorbance of the metallic nanoparticles in their aggregated state. By plotting the aggregation degree with concentration of potential binding partners, and using Michaelis-Menten equation such as that detailed in Example 3, binding constant K_d of the potential binding partners to the polypeptides may be determined.

[0067] As disclosed herein, a higher binding affinity means that the binding strength is stronger. A lower binding affinity means that the binding strength is weaker. Values of binding affinity may depend on the potential binding partner used.

[0068] Typically, when the binding affinity between a polypeptide and a potential binding partner is higher, a complex may be formed between the polypeptide and the potential binding partner, otherwise termed herein as a polypeptide-binding partner complex. The polypeptide-binding partner complex may bind to the surface of the metallic nanoparticles through one or more functional groups present on the polypeptide. In some embodiments, the polypeptide-binding partner complex may provide steric stabilization of the metallic nanoparticles against electrolyte-induced and/or thermal-induced aggregation. The binding partner stabilizes the polypeptide that is coated on the particles, such that the polypeptides do not denature upon addition of an electrolyte and/or when thermally treated.

[0069] The term “denature” as used herein refers to disruption of the conformation of a polypeptide molecule and associated loss of its function. For example, when only a polypeptide is added to the negatively charged metallic nanoparticles, the polypeptide may be slightly positively charged and is able to adsorb on the negatively charged nanoparticles. Therefore, upon addition of an electrolyte, the polypeptide provides a steric barrier to prevent aggregation of the metallic nanoparticles. The mutual compensation between electrostatic

destabilization and steric stabilization of the polypeptide on the particles leads to an intermediate particle stability.

[0070] In embodiments wherein the potential binding partner is able to bind to the polypeptide, the potential binding partner, otherwise termed as a binding partner in this instance, may bind to the polypeptide, thereby stabilizing the polypeptide against electrolyte-induced and/or thermal-induced denaturing of the polypeptide. As mentioned above, electrolyte-induced and/or thermal-induced denaturing of the polypeptide may take place in the form of polypeptides misfolding to a larger extent hence weakening of their protection effect on the metallic nanoparticles. Accordingly, using a method disclosed herein, presence or absence of interaction between polypeptide and potential binding partner may be determined.

[0071] In some embodiments, a polypeptide-binding partner complex is formed from a polypeptide containing one or more functional groups and a low molecular weight ligand, such as Quinobene, which refers to the tetrasodium salt of an organic tetrasulphonic acid, or Dawson polyoxometalate (POM).

[0072] In some embodiments, binding affinity between the polypeptide and the potential binding partner is low. A lower binding affinity may mean that less polypeptide-binding partner complexes are formed. In some embodiments, the potential binding partner does not interact with the polypeptide, and a polypeptide-binding partner complex is not formed. In such instances, the polypeptide and potential binding partner may provide lesser degree of stabilization or no stabilization of the metallic nanoparticles against electrolyte-induced and/or thermal-induced aggregation.

[0073] Generally, regardless of the binding affinity between polypeptide and potential binding partner, stabilization effect of a polypeptide-binding partner complex is greater than either polypeptide or potential binding partner alone, due to a combination of steric and electrostatic stabilization i.e. electrosteric stabilization, compared to a solution containing polypeptide or a combination of polypeptide and potential binding partner that does not interact with the polypeptide, as these may provide a lower degree of steric or electrostatic stabilization.

[0074] Instead, the potential binding partner may, in some embodiments, interact with the metallic nanoparticles to induce aggregation. Accordingly, to provide a more accurate analysis, the method disclosed herein may include removing excess and/or unreacted

potential binding partners from the mixture prior to addition of metallic nanoparticles. In so doing, this avoids interaction of the excess and/or unreacted ligands with metallic nanoparticles, that may induce aggregation of the metallic nanoparticles, and which may lead to an erroneous result being generated. The excess and/or unreacted potential binding partners may be removed by any suitable method such as spin filtration. In various embodiments, removing excess and/or unreacted potential binding partners from the mixture comprises spin filtering the mixture.

[0075] Not only may the method disclosed herein be used to detect the presence or absence of interaction of a polypeptide with a potential binding partner, it may also be used to quantify the binding efficiency of interaction of the polypeptide with the potential binding partner.

[0076] For example, depending on the binding efficiency in a sample compared to another sample, the degree of metallic nanoparticles aggregation may vary. A change of the degree of metallic particle aggregation may change, for example, absorbance of the mixture or size of the metallic nanoparticles aggregate.

[0077] Accordingly, in various embodiments, determining degree of aggregation of metallic nanoparticles is carried out by measuring at least one of (i) optical properties of the metallic nanoparticles, and (ii) size of metallic nanoparticle aggregates.

[0078] The differences exhibited may be correlated to the different binding efficiencies.

To determine the exact binding efficiency of a polypeptide and a potential binding partner present in a sample, results obtained from the sample may be compared with the results of a sample comprising a known binding partner binding to polypeptide with high affinity.

[0079] For example, binding efficiency of the polypeptide and potential binding partner may be determined by correlating at least one of (i) optical properties of the metallic nanoparticles, and (ii) size of the metallic nanoparticle aggregates to corresponding optical properties of metallic nanoparticles and size of metallic nanoparticle aggregates of another sample, wherein the other sample comprises a known binding partner that binds to the polypeptide with high affinity.

[0080] Methods to carry out such correlations are known in the art and may include, for example, generation of calibration curves obtained by measuring samples including a known binding partner binding to a polypeptide with high affinity.

[0081] As previously mentioned, determining interaction of a polypeptide with a potential binding partner may be achieved by measuring at least one of (i) optical properties of the metallic nanoparticles and (ii) size of metallic nanoparticles aggregates. The optical properties may be determined using a spectrophotometer or with a naked eye.

5 [0082] For example, aggregation may change the absorbance or the size caused by aggregation of multiple single nanoparticles. These changes may be measured using standard microscopic or spectroscopic methods. For example, optical properties such as absorbance may be determined with methods and devices known in the art, such as with a spectrophotometer. In various embodiments, determining optical properties with a
10 spectrophotometer is carried out with UV-Vis spectrometry at a wavelength range of about 400 nm to about 800 nm.

[0083] If the change of the optical properties is visible in the light wave range visible to humans, it is also possible to determine the differences with the naked eye. Using this method, determination the interaction of the polypeptide with the potential binding partner
15 may be carried out in a simple and fast manner by observing color change in solution without using analytical instruments, which is advantageous as the analytical instruments may be costly.

[0084] As mentioned above, some potential binding partners may induce aggregation of metallic nanoparticles. Therefore, in order to provide a more accurate analysis, excess and/or
20 unreacted potential binding partners may be removed from the mixture prior to determining the optical properties with a naked eye.

[0085] The size of the nanoparticle aggregates which form upon interaction of a polypeptide with a potential binding partner and induced by an electrolyte may be determined with transmission electron microscopy (TEM), or light scattering techniques or a coulter
25 counter for small particles.

[0086] Besides the above-mentioned, a method disclosed herein may also be used to determine temperature of aggregation of the polypeptide-binding partner complex. For example, metallic nanoparticles may be incubated with a polypeptide that has been heat treated at various temperatures. Due to the heat treatment, the protein starts to unfold thereby
30 weakening its shielding effects. As a result, metallic nanoparticles may aggregate extensively upon addition of an electrolyte. Therefore, by plotting ratio of absorbance of metallic nanoparticles in their aggregated state as compared to absorbance of the metallic

nanoparticles in their dispersed state against temperature, temperature of aggregation of the protein-ligand complex may be determined. An example of how this may be carried out is illustrated in Example 4.

[0087] The invention refers in a further aspect to a method for identifying a binding partner for a polypeptide of interest. The method comprises a) contacting the polypeptide and a candidate binding partner with metallic nanoparticles under conditions that allow polypeptide-nanoparticle conjugate formation; b) subjecting the conjugate to at least one of (i) an electrolyte and (ii) temperature increase; and c) determining degree of aggregation of the metallic nanoparticles, wherein a decreased aggregation of the metallic nanoparticles compared to a reference with no binding partner indicates binding of the polypeptide and the candidate binding partner. Examples of polypeptide, binding partner, and metallic nanoparticles have already been mentioned above.

[0088] Methods disclosed herein may be used in a myriad of applications, such as drug discovery, and detection of protein stability and ligand-associated protein stabilization which are useful, for example, in protein structural and functional genomics studies.

[0089] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0090] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0091] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

5

EXPERIMENTAL SECTION

[0092] A method to study small ligand-protein interaction by using noble metal nanoparticle, particularly gold nanoparticle (AuNPs), as reporter according to various embodiments is provided. This method exploits the salt and thermal stability of protein upon
 10 ligand binding and its regulation on dispersion/aggregation of AuNPs. Particularly, the assay according to embodiments relies on the following three principles, namely (1) proteins can coat on AuNPs; the as-formed protein-AuNPs conjugate (nanoparticle-protein corona) can withstand salt induced aggregation due to the steric and/or electrosteric protection by the protein layer; (2) changes in protein stability can affect its efficiency to protect AuNPs; and
 15 (3) ligand binding can enhance protein salt/thermal stability and such stabilization effect may be seen from AuNPs dispersion/aggregation profile.

[0093] AuNPs have unique optical properties that are highly sensitive to particle size and aggregation/dispersion status. When the particles aggregate (increasing size), the colloidal solution shows a characteristic color change from red to blue, associated with a plasmonic
 20 red-shift. It has been demonstrated herein for the first time that the enhanced stability of ligand-loaded proteins under salt and heat treatment may be detected colorimetrically and quantitatively by using AuNPs as reporters.

[0094] **FIG. 1(A)** shows a schematic diagram of the assay principle. Liganded-protein (I) has higher salt and thermal stability, thus providing better protection to AuNPs in the
 25 presence of salt and heat treatment. In comparison, the non-liganded protein tends to denature under high salt content (II) and thus gives moderate protection to AuNPs. Non-liganded protein is largely denatured under heat treatment (III), providing low protection to AuNPs. AuNPs aggregation profile may be measured by UV-vis spectroscopy, as shown in **FIG. 1(B)**.

30 [0095] Based on the above principles, with appropriate experimental design (**FIG. 2**), this assay is amendable for the following purposes: (i) Fast screening of positive and negative ligands for a protein (**Examples 1, 2, and 5**); (ii) Determining ligand-protein binding

constant (K_d) (**Example 3**); and (iii) Determining protein thermal stability (mid-point aggregation, or T_{agg}) with and without ligand binding (**Example 4**).

[0096] The Room Temperature (RT) assay (**FIG. 2A**) for fast screening of positive and negative ligands in various embodiments is made up of 5 experimental steps, namely:

- 5 1. Incubate protein-ligand (at molar ratio of 1:4) for 10 min at RT.
2. Spin-filter the incubation mixture to remove the excess or unreacted ligands (this step is Optional, see below).
3. Add the protein-ligand solution from Step 1 or the retained proteins/protein-ligand complexes from step 2 into AuNPs and incubate for 5 min.
- 10 4. Add sodium chloride (NaCl) (final concentration 86 mM), and immediately
5. Scan UV-vis spectra from 400 nm to 800 nm. The absorbance ratio (r) of A_{540}/A_{600} is calculated for quantitative measurement of AuNPs aggregation degree.

[0097] The Thermal Treatment assay (**FIG. 2B**) is similar to the RT assay, with an addition of 10 min heat treatment of the protein-ligand mixture or the retained protein/protein-ligand complex, prior to incubation with AuNPs. To determine the temperature of (protein) aggregation (T_{agg}), the heat treatment is performed in a range of 25 °C to 90 °C. To determine the binding constant, the thermal experiment was performed at T_{agg} of protein (i.e. 60 °C in this case), at a range of protein: ligand ratio of 1:0 to 1:25.

[0098] In both the RT and thermal treatment assays, step 2 is optional. It is necessary when the ligand itself can interact and aggregate AuNPs (**Example 5**).

[0099] Two transcription factors, i.e. FoxA1 and AP-2 γ , and 9 small ligands (**TABLE 1**) were used to prove the concept.

[00100]

TABLE 1: Low molecular weight ligands from the diversity, mechanistic and natural products libraries procured from the National Cancer Institute (NCI, <http://dtp.nci.nih.gov/index.html>)

Ligand No.	Name	Selectivity to FoxA1 and AP2 γ	MW
1	Quinobene	Positive	931
2	Dawson	Positive	3015
3	[[<i>(Z)</i> -(3-oxopyridin-2-ylidene)methyl]amino]thiourea (picolinaldehyde)	Negative	196
4	8-amino-10-phenylphenazin-2-one	Negative	287
5	2-bromo-1H-phenalen-1-one	Negative	259

6	Lomofungin	Negative	314
7	<i>N,N</i> -dimethyldaunorubicin	Negative	556
8	6H-Imidazo[4,1-de]acridin-6-one	Negative	415
9	Quinacrine	Negative	436

[00101] **Example 1: Fast screening of positive and negative ligands (for FoxA1) by exploiting protein salt stability at room temperature**

[00102] A transcription factor, forkhead box protein A1 (FoxA1), and 9 ligands (2 positive and 7 negative) were used in this example. FoxA1 (300 nM) and each ligand (2500 nM) were mixed for 10 min. The protein-ligand incubation solutions were subjected to filtration using Vivaspin 500 (50,000 MWCO) at 14,000 rpm for 10 min to remove the excess or unreacted ligands. The retained solution (containing proteins and/or protein-ligand complex) (20 μ l) was then incubated with AuNPs (75 μ l) for 5 minutes. 5 μ l 10% NaCl was added to the mixture (final NaCl concentration 86 mM) and the samples were analyzed with UV-vis spectroscopy immediately.

[00103] Prior to ligand screening, UV-vis spectra were recorded for AuNPs and FoxA1-coated AuNPs with and without NaCl (**FIG. 3A**) to confirm the fundamental principle (1) as mentioned earlier. In the absence of NaCl, citrates-stabilized AuNPs are well-dispersed due to the interparticle charge repulsion. The coating of FoxA1 on AuNPs is evidenced by the peak shift from 520 nm (bare AuNPs) to 540 nm (FoxA1-coated AuNPs). In the presence of NaCl, aggressive aggregation was observed for bare AuNPs. In contrast, FoxA1-coated AuNPs underwent moderate aggregation (peak shift to about 600 nm) under the same salt condition. The depleted aggregation of FoxA1-coated AuNPs relative to bare AuNPs is presumably due to electrosteric protection exerted by the protein layer. The remarkable aggregation of FoxA1-coated AuNPs under NaCl, relative to FoxA1-AuNPs without NaCl, may be attributed to the fact that salt can regulate the solubility and stability of the proteins coated on AuNPs.

[00104] In the subsequent ligand binding experiments (**FIG. 3B**), it was found that positive (affinity) ligand loaded proteins allow AuNPs to better retain the dispersion status. This was reasoned as the fact that ligand is capable of stabilizing the proteins against denaturants (high salt content). As a result, the proteins remain efficient to protect the particles and keep the particles dispersed. On the other hand, FoxA1 with negative ligands provides less effective electrosteric protection to AuNPs. The degree of aggregation of the particles was similar to FoxA1-AuNPs without any ligand. **FIG. 3C** is a summary of particle dispersion status

quantified as a ratio of absorbance (r) at the original peak of well dispersed FoxA1-AuNPs without NaCl (540 nm) to that of aggregated particles at 600 nm. The higher this r value is, the better dispersed the particles.

[00105] From **FIG. 3**, the positive and negative ligands from the plot of AuNPs dispersion status may be clearly identified. For example, the average r value for AuNPs with FoxA1 treated with seven negative ligands (lig 3 to 9) is 0.97 ± 0.06 . By setting $r = 1.14$ (average value + 3 standard deviation (S.D.)) as upper threshold, any unknown ligand with r below this value will be classified as negative ligand. As such this RT experiment allows one to quickly screen the positive and negative ligands for a given protein.

10 [00106] **Example 2: Improving screening sensitivity through thermal treatment of protein (FoxA1 and AP-2 γ)-ligand complex**

[00107] It has been demonstrated that AuNPs stabilized with thermally denatured antibodies are more susceptible to subtle environment changes than AuNPs coated with untreated antibodies. The colorimetric method disclosed herein is of notably high sensitive that it can detect subtle structural changes in protein that is undetectable by circular dichroism (CD).

[00108] Herein, it has been shown that by including thermal treatment to proteins with or without ligand binding, sensitivity of the assay may be further improved. Thermal treatment causes the proteins misfold to a larger extent and weakens its protection effect on AuNPs. Consequently, the color difference between the solutions with positive and negative ligands is more intense, allowing ligand-screening with higher sensitivity and even with naked eyes (**FIG. 4, photo**). Instead of showing peak broadening to 600 nm, new peak emerges at about 700 nm associated with decreased absorbance at 540 nm (**FIG. 4A**). The quantification of aggregation degree, measured as A_{540}/A_{700} , shows much larger difference between positive ligands (lig1 and lig2) and negative ligands (lig3 to lig5).

[00109] To demonstrate that the method disclosed herein may be generalized for other proteins, ligand screening was conducted on another transcription factor AP-2 γ protein. Three samples were prepared, namely protein without ligand, protein with positive ligand (lig 2) and protein with negative ligand (lig 3). After thermal-treatment, the protein-ligand incubation solutions were added to AuNPs, followed by addition of NaCl. The resulting UV-vis spectrum of AuNPs solution (**FIG. 5**) shows that the AP-2 γ -coated AuNPs shows a sharp single peak at 545 nm, without thermal treatment of the protein.

[00110] After thermal treatment of the protein solutions, AuNPs aggregation increased, but with a smaller degree of increase in the presence of positive ligand, relative to the negative ligand and non ligand. The UV-vis spectrum provides a quick assessment of the affinity of the tested ligand to AP-2 γ .

5 [00111] **Example 3: Determination of binding constant (K_d) of ligand-protein interaction using FoxA1-Dawson (lig 2) as an example**

[00112] As discussed in previous session, thermal treatment of protein enables the detection of ligand stabilization effect with a high sensitivity, by measuring the aggregation degree (A_{540}/A_{700} ratio) of AuNPs. By titrating fixed amount of FoxA1-coated AuNPs with
10 increased concentration of ligands (i.e. lig 2, Dawson), the absorbance at 700 nm (representing aggregated particles) was gradually decreased, associated with a gradual recover at 540 nm region (dispersed particles) (**FIG. 6A**).

[00113] When no significant change in UV-vis spectrum was observed, the ligand binding was assumed to be saturated, thus additional ligands exert no further effect on the FoxA1
15 proteins coated on AuNPs. By plotting the ratio of A_{540}/A_{700} (the degree of particle dispersion) vs. ligand concentration, the binding constant (K_d) may be calculated using Michaelis-Menten equation: $Y = R_{max} * C / (K_d + C)$. **FIG. 6B** shows the K_d measured for FoxA1-lig 2 (Dawson) using the above method.

[00114] To verify the accuracy of the assay disclosed herein for K_d measurement, surface
20 plasmon resonance spectroscopy (SPR, a golden standard method) was used to determine the K_d (**FIG. 6**) through titration of immobilized FoxA1 with increasing ligand concentration. The K_d values measured by SPR and current nanoparticle assay are 3344.51 and 2684.24 nM respectively. They are very close to each other.

25 [00115] **Example 4: Determination of temperature of aggregation (T_{agg}) of FoxA1 and its ligand complexes (i.e. thermal stability).**

[00116] Upon ligand binding, the free energy contribution from ligand increases the Gibbs free energy of unfolding (ΔG_u) of a protein. The ligand binding thus enhances the protein stability against heat, as evidenced by shifting of melting temperature of protein (T_m) to higher temperatures. By applying this principle in our assay, we can screen the T_{agg} of a
30 protein visually, similar as the midpoint of the aggregation process measured by differential static light scattering (DSLS).

[00117] To be specific, AuNPs was incubated with a protein treated at different temperature. When the protein starts to unfold due to thermal treatment, AuNPs aggregate extensively upon salt addition due to minimum surface coverage by the denatured proteins. By plotting the ratio of absorbance (r) vs. temperature, T_{agg} of a protein can thus be identified. Similarly, ligand-dependent protein stabilization may be measured from the thermal profile shift (**FIG. 8**).

[00118] The T_{agg} of FoxA1 determined by the colorimetric assay disclosed herein is 50 °C (**FIG. 8**), which is comparable to the value determined by DSLS (54°C) (**TABLE 2**). When incubated with negative ligand (i.e. lig 3), similar thermal profile as FoxA1 was observed. In contrast, the presence of positive ligands, i.e. lig 1 and lig 2, has significantly impeded the aggregation rate of AuNPs, which is indicative of the increased thermal stability or increased T_{agg} . The T_{agg} shift for FoxA1 with lig 1 to lig 3 measured by a colorimetric method disclosed herein and DSLS was summarized in **TABLE 2**.

[00119] **TABLE 2:** T_{agg} determined by DSLS and AuNPs colorimetric method for FoxA1 with positive and negative ligand.

Condition	T_{agg} measured by DSLS	T_{agg} by measured by AuNP colorimetric method
FoxA1 only	54	50
FoxA1 + lig 1 (positive)	64	80
FoxA1 + lig 2 (positive)	66.7	70
FoxA1 + lig 3 (negative)	54	50

[00120] **Example 5: Pre-screening of ligand-AuNPs interactions with naked eye**

[00121] Since protein coating to AuNPs and the consequent protection to AuNPs is essential for this assay, whether the chemical ligand itself can interact with AuNPs and cause particle aggregation is a concern for this assay to work (**FIG. 9A**). Experiment with the nine ligands listed in **TABLE 1** shows that some of them indeed can aggregate the particles (**FIG. 9**) and therefore invalid for further study. To solve this problem, a spin-filter step is included. The protein-ligand incubation solution was subjected to filtration with Vivaspın500 (50,000 MWCO) at 14,000 rpm for 10 min to remove the excess or unreacted ligands. The retained solution (containing proteins and/or protein-ligand complex) was then incubated with AuNPs for 5 minutes.

[00122] With this result, the disclosed assay will include a fast pre-screening step. A simple mixing of ligand with AuNPs can give a very fast visual result (FIG. 9C) of ligand effect on AuNPs. For the nine ligands tested, a noticeable color change relative to the bright red of the original AuNPs was observed for ligands 3, 7, 8, and 9. For these ligands, spin filtering step is thus required.

[00123] **Example 6: Discussion**

[00124] TABLE 3 provides a comparison summary of throughput, protein amount, and features of state of the art methods compared to a method disclosed herein.

[00125]

10 **TABLE 3:** Comparison of methods for ligand binding study.

Method	Through-put	Protein amount	Comparison of features	
differential scanning fluorimetry (DSF)	96, 384 wells	<5 µg/well	Independent of protein function	Sensitive to protein/ compound with fluorescence background
NMR	Low		More structural information of binding interaction	-Restricted to small proteins (<50 kDa) -pre-treatment is required (spin label, reporter ligands, etc)
differential static light scattering (DSLS)	384 well	<10 µg/well	Label free	-Protein must aggregate over short time scale -Not applicable to reversible protein denaturation
isothermal titration calorimetry (ITC)	Low	High µg to low mg	K_d	large protein amount
SPR	Low	Low µg	k_{on} , k_{off} , K_d	Require protein or ligand immobilization
CD	Low	High µg to low mg	Structural data	large protein amount
fluorescence polarization (FP)	96, 384 well	low µg/well	K_d	Labeled ligand; sensitive to ligand fluorescence
GFP based stability assay (GFP-Basta) ^a	96 well		No special instrument required	-GFP fusion to protein (protein modification) -Only for protein is less stable for GFP -fluorescent bleaching
Method disclosed herein	96, 384 well	~ 0.5µg/well	(i) Modification to protein and ligand (e.g. labelling with fluorophore etc) not required; (ii) Expensive equipment (CD, NMR, and SPR etc) not required; (iii) Constraints to molecular weight of ligand not present; (iv) Fast, e.g. results may be obtained within 1 h for 96 samples; (v) Small amount of protein	Removal of excess and/or unreacted ligands may be carried out for ligands that may intrinsically aggregate metallic nanoparticles.

			consumption (less than 1 μ g protein per test); (vi) Fast screening of positive and negative ligands; (vii) Quantitative determination of ligand-protein binding constant (K_d); (viii) Quantitative determination of protein stability (T_{agg}).	
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^a Mol. Biosyst., 2010, 6, 1285-1292

[00126] Most large equipment based methods (NMR, CD, SPR, ITC etc) suffer from drawbacks of relatively low throughput and high protein amount. Fluorescence-based methods usually require protein or ligand labeling and suffer from background fluorescence problem and fluorescence bleaching. Mass measurement based technique (i.e. SPR) has limited sensitivity to lower molecular weight ligand and requires protein/ligand immobilization. The GFP-based method requires protein treatment (fusion).

[00127] The method disclosed herein is based on a mix-and-test manner, without involving complicated sample preparation (labeling, immobilization) and expensive equipment. Advantageously, no modification to protein and ligand, such as label with fluorophore etc, is required. Further, unlike CD, NMR, and SPR etc, the method does not necessitate use of expensive equipment. There is no constraint to molecular weight of ligands used. The method allows for fast screening of positive and negative ligands. For example, analysis of 96 samples may be carried out within an hour, and only involves a small amount of protein of less than 1 μ g protein per test. Furthermore, quantitative determination of ligand-protein binding constant (K_d), and protein stability in the form of T_{agg} may be determined readily.

[00128] The simplicity, speed, cost-effective and amenability of this assay may thus offer a potential platform for high throughput screening. The assay disclosed herein requires no special equipment, extensive purification steps or fluorescent dyes. More importantly, it is also feasible for binding constant (K_d) and temperature of aggregation (T_{agg}) measurement in a tunable dynamic range.

CLAIMS

1. A method for determining interaction of a polypeptide with a potential binding partner, the method comprising
 - 5 a) providing a mixture comprising the polypeptide, the potential binding partner, and metallic nanoparticles;
 - b) subjecting the mixture to at least one of (i) an electrolyte and (ii) temperature increase; and
 - 10 c) determining the interaction of the polypeptide with the potential binding partner by determining degree of aggregation of the metallic nanoparticles.
- 15 2. The method of claim 1, wherein step a) comprises contacting the polypeptide with the potential binding partner, and subsequently contacting the resulting mixture with the metallic nanoparticles.
3. The method of claim 1, wherein step a) comprises contacting the polypeptide with the metallic nanoparticles, and subsequently contacting the resulting mixture with the potential binding partner.
- 20 4. The method of claim 1, wherein step a) comprises contacting the potential binding partner with the metallic nanoparticles, and subsequently contacting the resulting mixture with the polypeptide.
- 25 5. The method of claim 1, wherein step a) comprises simultaneous contacting of the polypeptide, the potential binding partner, and the metallic nanoparticles.
6. The method of any one of claims 1 to 5, wherein the polypeptide is selected from the group consisting of an antibody, a protein receptor, and a transcription factor.
- 30 7. The method of any one of claims 1 to 6, wherein the potential binding partner is a low molecular weight ligand or another protein.

8. The method of any one of claims 1 to 7, wherein the metallic nanoparticles are dispersed in the mixture of step a).
- 5 9. The method of any one of claims 1 to 8, wherein the metallic nanoparticles are noble metal nanoparticles.
10. The method of claim 9, wherein the noble metal nanoparticles comprise or consist of a noble metal selected from the group consisting of silver, gold and alloys of the
10 aforementioned materials.
11. The method of any one of claims 1 to 10, wherein the metallic nanoparticles each have a size in a range of about 10 nm to about 100 nm.
- 15 12. The method of any one of claims 1 to 11, wherein metallic nanoparticles are negatively charged metallic nanoparticles.
13. The method according to any one of claims 1 to 12, wherein excess and/or unreacted potential binding partners are removed from the mixture prior to addition of metallic
20 nanoparticles.
14. The method according to claim 13, wherein removing excess and/or unreacted potential binding partners comprises spin filtering the mixture.
- 25 15. The method according to any one of claims 1 to 14, wherein subjecting the mixture to temperature increase comprises heating the mixture at a temperature in the range of about 25 °C to about 90 °C.
16. The method of any one of claims 1 to 15, wherein the electrolyte is a salt.
- 30 17. The method of claim 16, wherein the salt comprises or consists of sodium chloride.

18. The method of claim 16 or 17, wherein concentration of the salt is in a range of about 0.01 M to about 0.5 M.
- 5 19. The method of any one of claims 1 to 18, wherein determining degree of aggregation of metallic nanoparticles is carried out by measuring at least one of (i) optical properties of the metallic nanoparticles, and (ii) size of metallic nanoparticle aggregates.
- 10 20. The method of any one of claims 1 to 19, wherein binding efficiency of the polypeptide and the potential binding partner is determined by correlating at least one of (i) optical properties of the metallic nanoparticles, and (ii) size of the metallic nanoparticle aggregates to corresponding optical properties of metallic nanoparticles and size of metallic nanoparticle aggregates of another sample, wherein the other sample comprises a known binding partner that binds to the polypeptide with high
15 affinity.
21. The method of claim 19 or 20, wherein the optical properties are determined with a naked eye or a spectrophotometer.
- 20 22. The method of claim 21, wherein determining optical properties with a spectrophotometer is carried out with UV-Vis spectrometry at a wavelength range of about 400 nm to about 800 nm.
- 25 23. The method of claim 21, wherein excess and/or unreacted potential binding partners are removed from the mixture prior to determining the optical properties with a naked eye.
24. The method of any one of claims 1 to 23, wherein the method is carried out at ambient conditions.
- 30 25. A method for identifying a binding partner for a polypeptide of interest, the method comprising

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- a) contacting the polypeptide and a candidate binding partner with metallic nanoparticles under conditions that allow polypeptide-nanoparticle conjugate formation;
- b) subjecting the conjugate to at least one of (i) an electrolyte and (ii) temperature increase; and
- c) determining degree of aggregation of the metallic nanoparticles, wherein a decreased aggregation of the metallic nanoparticles compared to a reference with no binding partner indicates binding of the polypeptide and the candidate binding partner.